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Capsaspora owczarzaki is an independent opisthokont lineage

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Transitions from unicellularity to multicellularity have occurred several times in the history of life. Two of the most conspicuous multicellular kingdoms, Metazoa and Fungi, form a monophyletic clade, known as the opisthokonts, which comprises some unicellular lineages, such as Choanoflagellata, Nucleariidae, Ichthyosporea (also known as the DRIPs or Mesomycetozoea), *Ministeria* and *Corallochytrium* [1,2]. Among these unicellular opisthokonts we should expect to find the closest extant relatives of the multicellular Metazoa and Fungi. Choanoflagellates have traditionally been considered to be closely related to metazoans [1–6] and the Ichthyosporea are proposed to be the next closest outgroup [5]. By contrast, the position of the nucleariids is controversial [7–10]. *Nuclearia sp.* (ATCC 30864), recently renamed as *Capsaspora owczarzaki,* a filose amoeboid symbiont of a pulmonate snail, has never been confidently assigned to any opisthokont lineage [7–9]. Here, we report large subunit ribosomal and actin sequences of *Capsaspora owczarzaki* and an actin sequence of the novel ichthyosporean species *Sphaeroforma arctica* [11]. We test the phylogenetic position of *Capsaspora owczarzaki*, and the phylogenetic position of the nucleariids within the opisthokonts.

A Bayesian analysis of a combined dataset made up of small and large ribosomal subunit nucleotide sequences as well as actin protein sequences was performed with all key taxa included. We employed a mixed model (nucleotide: GTR+Γ and protein: WAG+ Γ) with rates across sites and branch length parameters unlinked. Statistical support was assessed by Bayesian posterior probability (BPP) and Bayesian bootstrap support (BBS). This analysis shows that *Capsaspora owczarzaki* is not a nucleariid, but an independent opisthokont lineage closely related to choanoflagellates and ichthyosporeans. Moreover, *Nuclearia* branches as a sister-group to Fungi (Figure 1A). Surprisingly, the clade formed by *Capsaspora*, Choanoflagellata and Ichthyosporea, the CCI clade, appears to be most closely related to Fungi and Nuclearia , rather than to animals (BPP = 71%; BBS = 66%). This grouping, henceforth called the CCIFN group, contrasts with previous molecular phylogenies and morphological predictions in which choanoflagellates appear as a sister-group to metazoans [1–6]. However, a tree with the CCI clade as a sistergroup to metazoans, a CCIM grouping, appears also within the 95% Bayesian credible set and is, therefore, not rejected by this analysis. A possible explanation for the unexpected CCIFN grouping might be an artifactual attraction of long-branched metazoa by some of the very long-branch outgroups, such as *Entamoeba histolytica* and *Dictyostelium discoideum*. To test this, we ran an additional Bayesian analysis with branch lengths linked across the datasets. In theory, this could exacerbate the long-branch problem, since linked branch lengths are an average over all the genes and therefore likely a poor fit to each individual gene [12]. As expected, this resulted in an increasing support for the CCIFN group from 71 to 99% BPP (data not shown). Analyses were also performed using a covarion model, which allows the rate of substitution for sites to vary over time. With the covarion model (GTR + Γ + cov/WAG + Γ + cov), the CCIM clade is recovered (BPP = 94%; BBS = 50%; Figure 1B). This appears to be another clear example of the failure to account for covarion effects leading to positively misleading phylogenies [13].

In any case, the grouping of *Capsaspora* with choanoflagellates and *Nuclearia* as sister-group to Fungi is strongly supported in all analyses.

We conclude that *Capsaspora owczarzaki* is not a nucleariid, but an independent unicellular lineage more closely related to choanoflagellates and ichthyosporeans, and, thus, occupies an important position near the base of the Metazoa. *Capsaspora* is therefore a key taxon for future comparative genomic studies aimed at understanding the origin of metazoans. Furthermore, our results agree with other analyses (S.L. Baldauf, personal communication) that show that Nucleariidae is the unicellular protozoan group most closely related to Fungi.

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Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/14/22/R946/DC1/

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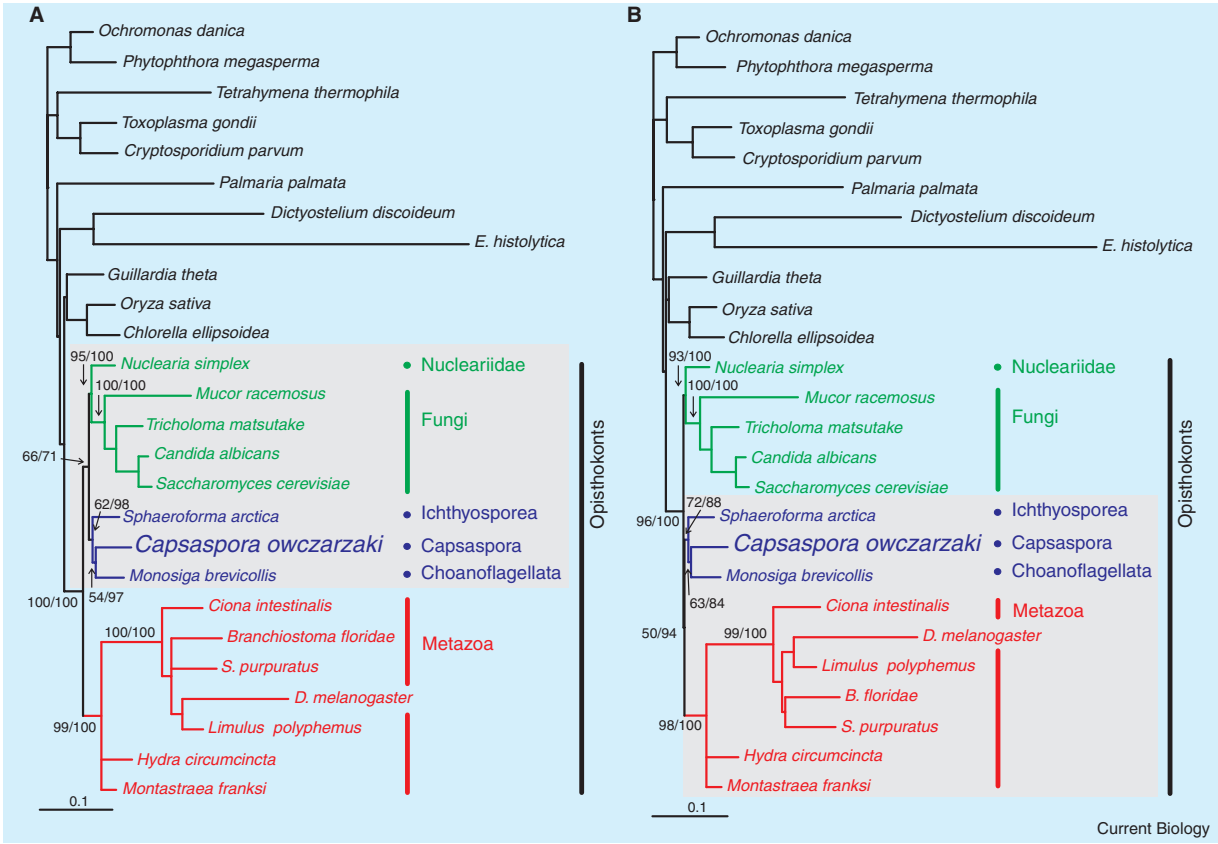

Figure 1. Capsaspora forms an independent ophisthokont lineage.

Bayesian Inference consensus tree of the combined (SSU+LSU+Actin) datasets. (A) GTR+Γ and WAG+Γ models of evolution. The group indicated in shaded box is the 'CCIFN' group. (B) GTR+Γ+cov and WAG+Γ+cov models of evolution. The shaded group is the 'CCIM' group. Values above key nodes refer to the BBS and the BPP, respectively (shown as percentage). Both analyses were done using Mr. Bayes3 [14] by three independent runs of a mixed model (nucleotide and protein) of all three partitions (SSU, LSU, and actin), each one with 500,000 Markov Chain Monte Carlo generations with four simultaneous chains (trees from the first 150000 generations were discarded as burn-in). Rates across sites (8 categories) and branch lengths were unlinked, i.e., each dataset has its own α-shape parameter and set of branch lengths. Statistical support was assessed by Bayesian posterior probability (BPP) and by bootstrapping (100 replicates) the datasets individually, reconcatenating them and finding the tree of maximum posterior probability (MAP) for each replicate and the frequency of splits recovered in the MAP trees from all the replicates (referred as Bayesian bootstrap support or BBS). Bayesian and ML analyses were also performed with the actin and ribosomal datasets alone (Supplemental Data). GenBank accession numbers: AY724688, AY724689, AJ780965.

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